K.W., "Digital PCR", Proc. Natl. Acad. Sci. U.S.A. 96:9236-9241 (1999). This technique involves the fewest number of PCR cycles required for dependable results reported to date.

[0018] Ethanol is a preferred tissue fixative because it precipitates antigens and does not cause DNA crosslinking, as does formalin. Following ethanol fixation, tissue is embedded in paraffin and sliced in thin sections by standard procedures. Routine pathology sections conventionally are between 4 μm and 5 μm in diameter. When using this diameter, tissue sections consist not only of undamaged sections, but also contain some damaged nuclei, resulting in allele dropout. To enhance the possibility of obtaining undamaged nuclei and reducing the risk of dissecting damaged cell nuclei, large sections, e.g., about 6 μm or greater are preferred. Steam heating using an EDTA buffer has been found to yield reliable immunohistochemical staining and intact DNA. Taylor, C.R., Shi. S.R., Cote, R.J., "Antigen retrieval for immunohistochemistry status and need for greater standardization", Applied Immunohistochemistry 4:144-146 (1996). Suitable conditions include a buffer containing 1mM EDTA (pH 8.0) at 96 to 100° C and heating for 5 minutes. Alternatively, steam heating can be performed using 20 mM HEPES/1mM EDTA buffer (pH 8.1) with a pK much less affected by high temperature. Using these process enhancements, the size of the single stranded DNA has been determined to have an average length of 20 kb.

[0019] Cells that accumulate p53 or have altered levels of a protein which is the product of a gene whose expression is regulated by p53 may be identified by immunohistochemical staining. Monoclonal antibodies that may be used for this purpose are available commercially. See Examples, infra. Because mutant p53 accumulates in cells, staining for this protein is useful for

identifying cells in which mutations have occurred. The p53 protein regulates the expression of a number of other genes, including PCNA, mdm2 and vEGF. Thus the levels of the proteins that are the products of these genes often are altered in cells containing mutant p53. For example, p53 down regulates PCNA, and mutant p53 may result in accumulated levels of PCNA in cells. The levels of other proteins under p53 control may be increased or decreased, depending on the mechanism of the control. The use of the levels of expression of one or more of such secondary proteins assists in differentiation between cells having enhanced levels of wt p53 resulting from natural physiological induction and the cells of interest having accumulated levels of mutant p53.

A2 cont.

[0021] Mutation analysis, including determination of mutation load, advantageously is determined by amplification and analysis of DNA from a single cell. The DNA is amplified by any procedure that efficiently reproduces DNA from the low template concentrations obtained from a single cell. A preferred amplification procedure, referred to herein as "Stimulated PCR," has been found to yield sufficient DNA for sequence analysis using as few as 40 cycles of amplification. This PCR process differs from known processes in that it substantially reduces the threshold effect of the template concentration on PCR efficiency. Additional advantages of Stimulated-PCR may include: inhibition of absorbance of the template to the tube surface; protection against minimal DNAase activity; addition of false priming sites for spurious extensions; activation by binding DNA polymerase; or direct stimulation of extension by DNA polymerase. Stimulated PCR is described in detail in the Examples, infra. In general, the technique is characterized by the use of a combination of a Taq polymerase High Fidelity™ and Taq DNA polymerase and by the incorporation of mouse genomic DNA having an average size of more

A3

than about 20 kb. The addition of mouse genomic DNA allows a wider range of annealing temperatures, a wider range of primer concentrations, less primer dimer formation, and higher product yield. Similar effects were observed by adding or supplementing bovine serum albumin (BSA), probably because BSA protein assists in keeping DNA polymerases in active forms.

A3 CDO4.

[0022] Changes in the use of High Fidelity™ enzymes, commercially available and utilized by those of skill in the art, improve amplification yields. The highest yields are found when the High Fidelity™ enzymes are used in higher amounts than those typically used, e.g., at about 4-fold the amount recommended by the manufacturer (2.5 U Taq/GB-D DNA polymerases per 25 µl of reaction). Additionally, mixing 1 U of PLATINUM Taq with 1 U of the High Fidelity™ enzymes, which increases the unit ratio of Taq to GB-D by 2 fold, behaves better than the High Fidelity™ enzymes alone, indicating that not only the total units, but also the relative ratios of the enzymes are important. Another improvement that may be used to increase the fidelity of the amplification and to minimize primer-dimer formation is the incorporation of a Taq antibody to inactivate Taq DNA polymerase at room temperature. This improvement is used in so-called HOTSTART PCR. In the present method, it has been effective in preventing primer dimer formation using 40 to 45 cycles.

A4

[0025] b. T_m of the primer was estimated by the nearest neighbor method at 50 mM KCl and 250 pM DNA and T_m of the PCR segment was estimated by the formula of Wetmur: $T_m^{product} = 81.5 + 16.6 \log[K^+ = 0.05\ M] + 0.41\ (\%G + \%C) = 675/length$.

A5

[0036] The PCNA antibody (Ab-1 monoclonal mouse IgG antibody) (Oncogene Calbiochem) was used in a concentration of 1:4000; the p53 antibody (mouse monoclonal antibody DO7) (Novocastra) was used

in a concentration of 1:100. The tissue sections were double stained immunohistochemically for p53 and PCNA. Cells testing positive showed p53 positive nuclear staining (bright red), PCNA positive cytoplasmatic staining (light brown) or both PCNA and p53 positive staining (reddish brown).

A5 cont.

[0037] The single cells were manually microdissected using an inverted microscope (Nikon TMS) and a mechanical micromanipulation system (Sutter Instruments). A tungsten needle was manipulated through a joystick. The microdissected cell was then picked up manually with a new 27 G ½" needle, and transferred into a 0.2mL PCR tube containing 5µl digestion buffer: #3 High Fidelity™ buffer without Magnesium, 2 mg/ml Proteinase K (Qiagen), 3% Tween-20 detergent and 0.2mM EDTA (pH 8.0). The single cell was digested at 50°C for 16 hr and after the digestion, Proteinase K was inactivated at 90 °C for 10 minutes. This single cell was then amplified by Stimulated PCR as set forth below in Example II.

[0039] In order to detect mutations in the single cell chosen for microdissection isolation from the paraffin-embedded tissue, the single cell was subjected to the Stimulated PCR technique. In preparing for the Stimulated-PCR used to amplify the single cell mutations, primer selection is important. Here, all primers were designed and analyzed with Oligo 5 software (National Biosciences).

A6

T^m of the primer was estimated by the nearest neighbor method at 50 mM KCl and 250 pM DNA and Tm of the PCR segment was estimated by the formula of Wetmur: $T^m_{product} = 81.5 + 16.6 \log[K^+=0.05M] + 0.41(\%G + \%C) - 675/length$. Wetmur, J.G., "DNA probes: Applications of the Principles of Nucleic Acid Hybridization", Critical Rev. in Biochem and Mol Biol. 26:227-259 (1991), the disclosure of which is incorporated herein by reference. The criteria for specificity included high-specificity with low base-pairing stability at the 3' end, no primer-dimer or hairpin formation more than 3 bases at the

3′ end, no homo- or repeat-sequence at the 3′ end, and no false priming site more than 7 bases at the 3′ end for any strand and any segment. The primer also had no false priming site on the mouse p53 gene to generate spurious products. The primers used are shown in Table 1, above. For Stimulated PCR, primer GCCGTCTTCCAGTTGCTTTATCTGTTCACT (SEQ. ID. NO. 1) was used in conjunction with either CCTGATGGCAAATGCCCCAATTGCAGGTAA (SEQ. ID. NO. 2) or GTCAAGTAGCATCTGTATCAGGCAAAGTCATAG (SEQ. ID. NO. 3). The results of a 2 kb region of the p53 gene amplification with 0.6 µl of p53(12983)30D (SEQ. ID. NO. 1) and p53(15036)33U (SEQ. ID. NO. 3) from genomic DNA of twelve single microdissected cells for 40 cycles are shown in Figure 2.

[0040] The PCR mixtures contained a total volume of 25 µl: human genomic DNA from a dissected single cell; #3 Expanded High Fidelity buffer (Boehringer Mannheim); 3.5 mM $MgCl_2$; 500 µM of each dNTP; 2% DMSO; 0.2 to 0.6 µM of each of primers; a mixture of 1.25U of PLATINUM Taq DNA polymerase High Fidelity (Taq/GB-D)/1.25U of PLATINUM Taq DNA polymerase (GIBCO BRL); 5 µg of BSA and 25 ng of mouse genomic DNA with the average size more than 20 kb. The cycling conditions included denaturation at 92 °C for 12 seconds, annealing at 60 °C for 20 seconds, and elongation at 68 °C for 2 minutes for 40 or 45 cycles with a Perkin Elmer GeneAmp PCR system 9700. An additional 20 seconds of denaturation time proceeded the first cycle. Two to 4 µl of the PCR product was electrophoresed through a standard 1% agarose gel and then the gel was stained with ethidium bromide for UV photography by a CCD camera (Bio-Rad Gel Doc™ 1000) and Multi-Analyst™ software (version 1.1). Another nested or half nested PCR was performed for 12 to 15 cycles to obtain more product. For this additional half-nested PCR, primers CCTGATGGCAAATGCCCCAATTGCAGGTAA (SEQ. ID. NO. 2) and TGTTCACTTGTGCCCTGACTTTCAACTCTG [SEQ. ID NO. 4] were used.

A6 cont.

[0041] The PCR product was purified for two rounds using Microcon® 100 (Amicon) to remove the unincorporated primer and primer dimers. Standard sequence analysis was performed using ABI 377 fluorescence sequencer and BigDye™ terminator chemistry with AMPLITAQ FS DNA polymerase (PE Applied Biosystem). The primers used during the sequencing process were: TGCCCTGACTTTCAACTCTGTCTC (SEQ. ID. NO. 5); AGGGTCCCCAGGCCTCTGAT (SEQ. ID. NO. 6); GGCCACTGACAACCACCCTTAA (SEQ. ID. NO. 7); AGGTCTCCCCAAGGCGCACT (SEQ. ID. NO. 8); GGGGCACAGCAGGCCAGTGT (SEQ. ID. NO. 9); GGAGAGACCGGCGCACAGA (SEQ. ID. NO. 10); and CGGCATTTTGAGTGTTAGACTGGA (SEQ. ID. NO. 11).

A6 Cont.

[0044] a. All of the above were identified from 15 normal nontumorous colon cells with *p53* and PCNA double straining. The missense mutations are either at a conservative site or are found in the *p53* mutation database (http://www.iarc.fr/P53/index.html). Mutations were also identified in other normal cells of breast, lung, kidney and gallbladder.

A7

In the Claims:

Please cancel claims 16 and 17, amend claims 1-15, 18-19 and 25 and insert new claim 26 as indicated. A marked-up version of the amended claims is attached to this amendment.

1. (Amended) A method for determining mutation load which comprises identifying a somatic cell that contains accumulated levels of p53, amplifying by PCR DNA of said identified somatic cell and determining the frequency or nature of mutations in said amplified DNA.

A8